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13. SUPPLEMENTARY NOTES

14. ABSTRACT The prolactin receptor (PRLr) is a cytokine receptor that binds prolactin (PRL) via its extracellular domain and consequently signals through the Jak2/Stat5 pathway. The canonical function of PRLr in this pathway is to serve as a membrane bound intermediary between PRL and downstream secondary messengers. However, recent data from our lab has revealed that PRLr localizes to the nucleus of breast cancer cells tissue. Preliminary experiments also demonstrated that PRL initiates nuclear localization of PRLr and the interaction between nuclear PRLr and the transcription factor, Stat5a. We therefore hypothesized that PRLr functions as a Stat5a co-activator. Investigation of this hypothesis via ChIP revealed that PRLr binds to the Stat5 responsive CISH promoter and reporter assays demonstrated that PRLr promoter engagement results in CISH activation. As these results pointed to a role for the PRLr in transactivation, we utilized the Gal4 assay, to determine that residues 404-448 of PRLr intracellular domain were necessary and sufficient for transactivation. Consequentially, mutation of two conserved residues Y406, and D411 disrupted transactivation, demonstrating the necessity of these residues for PRLr specific gene activation. In addition, immunoprecipitation analysis led us to reveal a novel finding; that the PRLr transactivation domain is necessary for recruitment of the chromatin modifying protein, HMGN2. Subsequent studies demonstrated that HMGN2 is also recruited to the Stat5a-driven CISH promoter in a PRL dependant manner. In addition, overexpression of HMGN2 enhances Stat5a driven CISH expression, while stable knockdown of HMGN2 impairs this expression. HMGN2 knockdown also prevents binding of the PRLr to the CISH promoter suggesting that HMGN2 is required to PRLr promoter binding. Since HMGN2 possesses no sequence specificity, our findings suggest that nuclear PRLr may serve as the adaptor molecule that tethers HMGN2 to Stat5a, resulting in full transcriptional activity of Stat5a. Ongoing research will seek to determine the mechanism of HMGN2 recruitment, as well as the phenotypic effects of transactivation-deficient PRLr.

15. SUBJECT TERMS
Prolactin prolactin receptor, Sta5a, breast cancer

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Table of Contents

Part I - Introduction

The prolactin receptor (PRLr) is a cytokine receptor that binds prolactin (PRL) via its extracellular domain and consequently signals through the Jak2/Stat5 pathway. The canonical function of PRLr in this pathway is to serve as a membrane bound intermediary between PRL and downstream secondary messengers. However, recent data from our lab has revealed that PRLr localizes to the nucleus of breast cancer cells tissue. Preliminary experiments also demonstrated that PRL initiates nuclear localization of PRLr and the interaction between nuclear PRLr and the transcription factor, Stat5a. We therefore hypothesized that PRLr functions as a Stat5a co-activator. Investigation of this hypothesis via ChIP revealed that PRLr binds to the Stat5 responsive CISH promoter and reporter assays demonstrated that PRLr promoter engagement results in CISH activation. As these results pointed to a role for the PRLr in transactivation, we utilized the Gal4 assay, to determine that residues 404-448 of PRLr intracellular domain were necessary and sufficient for transactivation. Consequentially, mutation of two conserved residues Y406, and D411 disrupted transactivation, demonstrating the necessity of these residues for PRLr specific gene activation. In addition, immunoprecipitation analysis led us to reveal a novel finding; that the PRLr transactivation domain is necessary for recruitment of the chromatin modifying protein, HMGN2. Subsequent studies demonstrated that HMGN2 is also recruited to the Stat5a-driven CISH promoter in a PRL dependant manner. In addition, overexpression of HMGN2 enhances Stat5a driven CISH expression, while stable knockdown of HMGN2 impairs this expression. HMGN2 knockdown also prevents binding of the PRLr to the CISH promoter suggesting that HMGN2 is required to PRLr promoter binding. Since HMGN2 possesses no sequence specificity, our findings suggest that nuclear PRLr may serve as the adaptor molecule that tethers HMGN2 to Stat5a, resulting in full transcriptional activity of Stat5a. Ongoing research will seek to determine the mechanism of HMGN2 recruitment, as well as the phenotypic effects of transactivation-deficient PRLr.

Part II – Proposed Aims
Three specific aims were proposed in this pre-doctoral award.

Specific Aims
(1) Evaluate the function of the PRLr as a potential transcription factor and/or Stat5a co-activator.
(2) Determine the molecular mechanism of PRLr nuclear localization.
(3) Analyze the role of nuclear PRLr in the pathogenesis of human breast carcinoma.

Part IIII - Statement of Work
The tasks to achieve the specific aims above are covered in the statement of work as outlined below. Items that had to be completed in the first year of the proposal are **indicated in bold.**

***Specific Aim 1.* Evaluate the function of the PRLr as a potential transcription factor and/or Stat5a co-activator (Months 1-20):**

Experiment 1.1 – What domains of PRLr are necessary and sufficient for transactivation?
   a. Months 1-4: Construct Gal4-PRLr truncation mutants to elucidate the transactivation domain of PRLr
   b. Months 4-7: Perform luciferase experiments with constructs in 293, MCF7 and T47D cells
   c. Months 7-12 Fine map the transactivation domain of the PRLr.

Some of the above data were reported in the 2009 Summary of Work. More detailed work has since been performed and the results of this are reported.

Experiment 1.2 - Does PRLr bind DNA in a Stat5a independent manner
   a. Months 6-7: Acquire biotinylated probes for EMSA
   b. Months 7-8: Perform EMSA studies using nuclear extracts
   c. Months : 8-10: Perform *in vitro* transcription/translation of PRLr and Stat5a
   d. Months 10-20: Perform EMSA studies on using *in vitro* transcribed/translated proteins

We have identified that the nuclear protein HMGN2 binds to both the PRLr transactivation domain, and Stat5a. We therefore performed experiments that ask the question: What is the role of HMGN2 in Stat5a driven gene expression?

Specific Aim 2. Determine the molecular mechanism of PRLr nuclear localization (Months 1-24):

Experiment 2.1 - Does PRLr sequence KPKK facilitate PRLr and Stat5a nuclear localization though a "classic" nuclear import pathway?
   a. **Months 1-4: Construct muPRLr (KPKK mutation) construct**
   b. **Months 4-8: Perform transfections of wt and mutant PRLr constructs in CHO cells**
   c. **Months 4-8: Perform western blots on cytoplasmic and nuclear extracts of CHO transfectants**

We have performed these experiments which were reported last year, but have since seen that mutation of the PRLr NLS has no effect of its nuclear localization. However, we have also identified two specific residues that are necessary for PRLr transactivation function. Therefore, we are utilizing a PRLr transactivation deficient construct to ask the question: What are the mechanistic and functional effects of mutation of the PRLr transactivation domain?

Experiment 2.2 - Is Jak2 tyrosine kinase necessary for initiating PRLr nuclear localization?
   a. Month 8:   Acquire Jak2 inhibitor (AG-490) and Jak2 siRNA
   b. Months 9-10: Perform drug treatment and on T47D cells
   c. Months 9-10: Perform western blot analysis on T47D nuclear and cytoplasmic lysates
            -Probe for PRLr, controls
   d. Months 10-12: Optimize si-RNA knockdown of Jak2
   e. Months 12-14: Perform western blot analysis of T47D cells with Jak2 knockdown
            -Probe for PRLr, controls

We have chosen to hold off on these experiments so that we can explore other aims more rigorously.

Experiment 2.3 - Is nuclear PRLr derived from the cell surface?
   a. **Months 14-16: Optimize biotinylation conditions**
   b. **Months 16-24: Perform biotinylation experiments in nuclear/cytoplasmic lysates.**

These results were reported in the 2009 Summary of work, but since we have performed a more thorough timecourse of this experiment. The results of this study are shown in this report.

Specific Aim 3. Analyze the role of nuclear PRLr in the pathogenesis of human breast carcinoma (Months 12-36):

Experiment 3.1 – Does nuclear targeted PRLr have the ability to induce neoplastic abnormalities in vitro?
   a. **Months 12-16: Create lentiviral constructs**
   b. **Months 16-20: Create stable cell lines**
   c. Months 20-24: Perform 3-D matrigel assay
   d. Months 22-24: Perform proliferation assay
   e. Months 24-28: Perform Growth on soft agar assay

We have identified the PRLr transactivation domain and HMGN2, the protein that is recruited to the PRLr transactivation domain, and is involved in chromatin regulation. We have therefore generated the following cells lines to study functional effects:

1. **HMGN2 knockdown**
2. **PRLr knockdown**
3. **wt PRLr overexpression**
4. **transactivation-deficient PRLr overexpression (PRLr Y406FD411A)**

As the PRLr knockdown shRNA targets the 3'UTR, we are also in the process of making rescue cells lines:
1. PRLr knockdown, wt PRLr overexpression
2. PRLr knockdown, Y406FD411A overexpression

Experiments 3.2 – Does a nuclear targeted PRLr have the ability to induce in vivo *tumor formation?*
   a. Months 24-28: Inject nude mice with stable cell lines (created in experiemnet#1 above)
   b. Months 28-36: Analyze tumor formation and metastasis in live mice and at experimental endpoints
   c. Months 28-26: Analyze histology of tumors, analyze markers such as Cyclin D1 by RT-PCR and western blot

Part IV - Results

Specific Aim 1. **Evaluate the function of the PRLr as a potential transcription factor and/or Stat5a co-activator:**

Identification of a PRLr transactivation domain.

Preliminary experiments have demonstrated that a portion of the PRLr intracellular domain, when fused to a Gal4 DNA binding domain, is able to strongly transactivate a reporter that contains 5 Gal4 binding sites upstream DNA encoding luciferase. This data suggests that PRLr ICD possesses a transactivation domain. Thus, the goal of this experiment is to determine which domain within the PRLr intracellular domain and which residues are responsible for PRLr transactivation function.

Over the part year we have identified the PRLr transactivation domain within residues 404-448. When aligned across mammalian species, we identified 4 highly conserved residues which were mutated to determine importance within the PRLr transactivation domain (Figure 1A). While mutation of Y406 or D411 alone decreased Gal4 transactivation, combining these mutations completely abrogated transactivation function (Figure 1B, 1C).

These data led us to ask the question; what might the PRLr be recruiting to chromatin to enable transcriptional activation? To answer this question, we revisited previous yeast two hybrid data in which the goal of the experiment was to identify proteins that bound specifically to the PRLr intracellular domain.

One protein of interest that was identified from this experiment was the nucleosome binding protein HMGN2. The HMGN family of proteins is known to modulate the chromatin structure in vertebrates. These proteins bind with higher affinity to nucleosomes then to double stranded DNA and their to nucleosomes also alters the posttranslational modification of nucleosomal histones at several distinct sites. The binding of

Figure 1. Y406FD411A abrogates Gal4 transactivation. *A.* Sequence analysis of the previously identified PRLr transactivation domain revealed a conserved domain with the N-terminus of this region with 4 highly conserved residues.   *B.* Mutation of(Y406→F and D411 →A) co mpletely ablated PRLr-ICD activity within either the Gal4-DBD-404-448 PRLr chimera or a Gal4-D BD-PRLr-ICD construct (not shown).

these proteins to nucleosomes therefore results in transcriptional stimulation or inhibition. HMGN2 possesses no DNA sequence specificity since it binds generically to nucleosomes. It is therefore hypothesized that the targeting of HMGN proteins to DNA is mediated by an adapter protein that confers DNA sequence specificity.
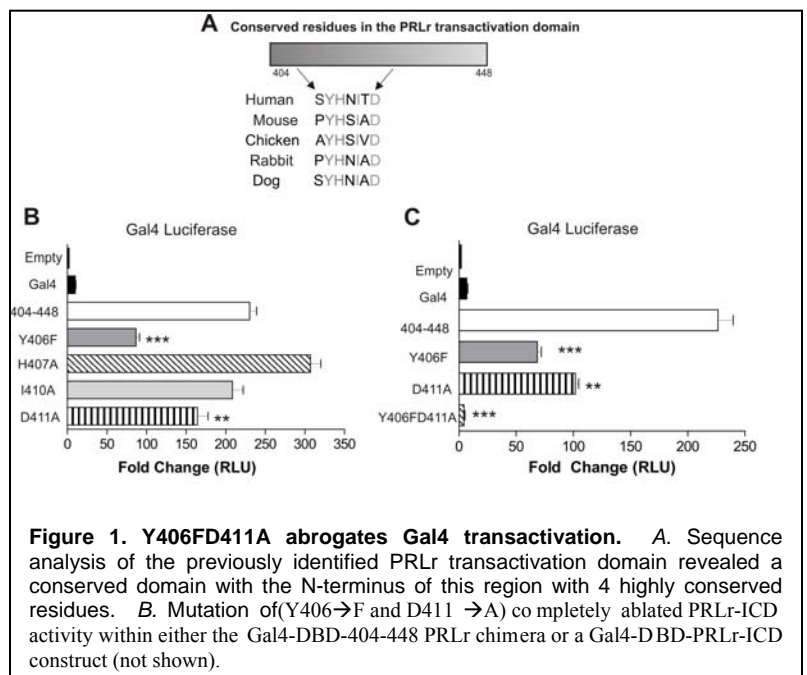
The PRLr transactivation domain, but not the transactivation mutant binds to HMGN2

We first sought to confirm that HMGN2 the yeast-two hybrid findings – that HMGN2 binds to the PRLr intracellular domain. To do this we utilized the heterologous Gal4 system, since within this system the PRLr is specifically targeted to chromatin. As the role of a transactivation domain is to activation transcription by interacting with other co-factors/transcriptional regulators, we hypothesized that if PRLr and HMGN2 do indeed bind, the PRLr transactivation domain would be the site of their interaction. Since Y406 and D411A are critical for PRLr transactivation function, we also reasoned that the TAD mutation (Y406F411A) would not have the ability to bind HMGN2.

As expected, HMGN2 strongly bound to the PRLr transactivation domain (404-448), but not the mutated TAD (Y406DD411A) or a region just outside of the PRLr TAD (485-517), (Figure 2A).

Endogenous PRLr and Stat5a bind to HMGN2 in T47D breast cancer cells

When we examined endogenous binding of the PRLr and HMGN2 in T47D breast cancer cells by co-immunoprecipitation analysis, we again confirmed binding. In addition, analysis also revealed that Stat5a binds to HMGN2, supporting the notion that the PRLr functions a Stat5a-coactivator, and recruits HMGN2 to the transcriptional complex.

Figure 2. The high mobility group protein HMGN2 binds to the PRLr transactivation domain. *A.* Co-immunoprecipitation analysis reveals that HMGN2 associates with the PRLr-404-448 domain but fails to associate with the transcriptionally-inactive, double point mutant PRLr-404-448 construct *B.* Co-IP analysis reveals that HMGN2 associates with endogenous PRLr and Stat5a in T47D cells.

Chromatin immunoprecipication of HMGN2 and PRLr to the CISH promoter

To determine if HMGN2 is bound to the PRLr/Stat5a chromatin complex on the CISH promoter, we performed
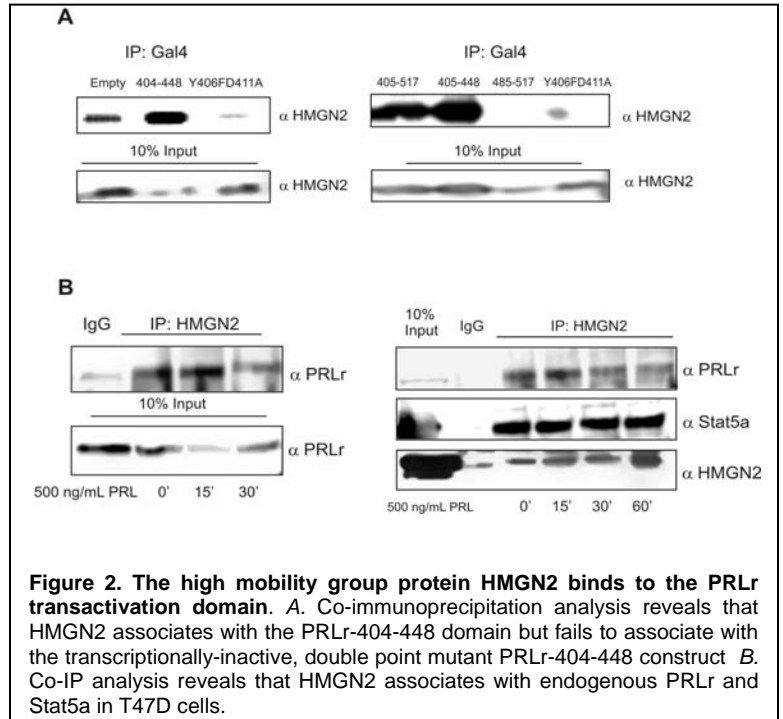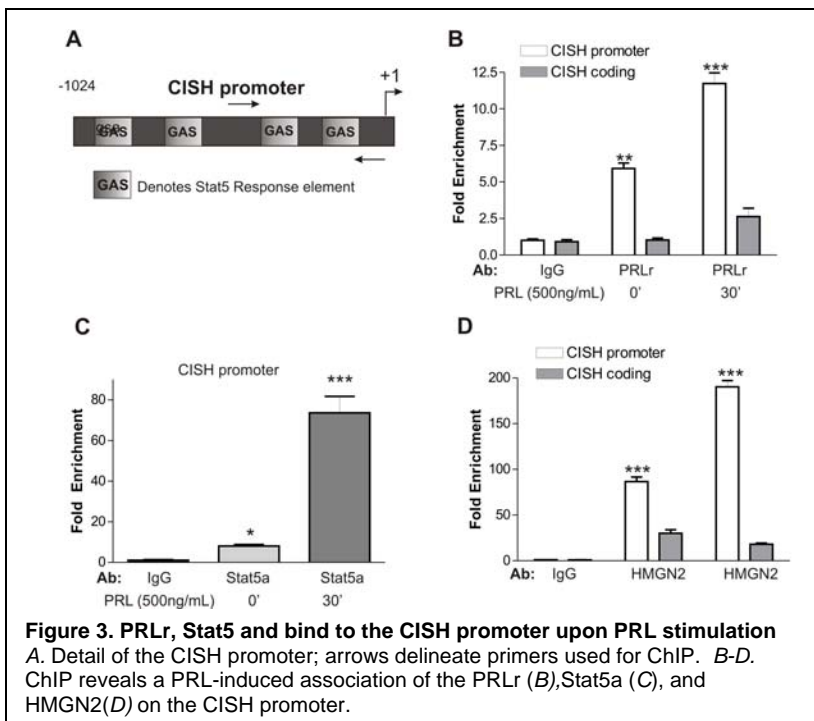

Figure 3. PRLr, Stat5 and bind to the CISH promoter upon PRL stimulation *A.* Detail of the CISH promoter; arrows delineate primers used for ChIP. *B-D.* ChIP reveals a PRL-induced association of the PRLr (*B*),Stat5a (*C*), and HMGN2(*D*) on the CISH promoter.

chromatin immunoprecipitation analysis using primers that overlap a Stat5a consensus sequence(Figure 3A). As previous data has demonstrated, PRLr binds strongly to the CISH promoter upon PRL stimulation (Figure3B). Similarly, we observed HMGN2 recruitment to the CISH promoter in a PRL-driven manner (Figure 3D). As a negative control in this assay primers that overlapped the CISH coding region were used. As shown in Figure 3, PRLr, Stat5a and HMGN2 bind specifically to the CISH promoter, while binding to the CISH coding region is negligible.

We then asked the question, does this binding result in enhanced transcriptional activation of CISH? Overexpression of HMGN2 in MCF7 cells significantly enhanced both basal and PRL-induced CISH-reporter activity. In addition, overexpression of PRLr and HMGN2 together produced an additive effect on CISH-reporter activity (Figure 4A-B).

Figure 4. HMGN2 overexpression significantly enhances PRL-driven CISH expression. (A) Expression of HMGN2 or PRLr in MCF7 cells potentiates PRL-induced CISH-luciferase expression, while **(B)** co-expression of wt PRLr and HMGN2 induces an additive effect on reporter activity. **(C)** The transactivation deficient mutation PRLr Y406FD411A impairs CISH reporter activity.

As demonstrated by co-immunoprecipitation analysis, HMGN2 cannot bind to the Gal4-PRLr transactivation domain mutant. We therefore postulated that introducing this mutation into full length PRLr would prevent PRL-induced CISH transcriptional activity. As expected this mutation significantly decreased CISH reporter activity over wt PRLr levels (Figure 4C). These results suggest that the PRLr transactivation domain, and HMGN2 play a crucial role in the activation of PRL-induced gene expression.

Specific Aim 2 – Determine the molecular mechanism of PRLr nuclear localization. *It has been demonstrated that the PRLr binds to the CISH promoter upon PRL stimulation. Therefore, we hypothesize that nuclear PRLr is derived from the cell surface, and does not exist as an independent pool of non-membrane targeted protein. Given that PRL binds PRLr on the cell surface, it is also conceivable that PRL may also be an initiating factor in PRLr nuclear trafficking. This set of experiments will therefore serve a dual purpose: 1) to demonstrate that PLRr is shuttled from the cell membrane to the nucleus and 2) to determine how PRL stimulation may initiate or regulate this event.*

Cell Surface PRLr localizes to the nucleus upon PRL stimulation

In the 2009 summary we demonstrated by biotinylation of cell surface proteins that nuclear PRLr is indeed derived from the cell surface, and this nuclear localization is initiated by PRL. Since that time, we have performed a more thorough timecourse as shown in Figure 5.

Specific Aim 3 – Analyze the role of nuclear PRLr in the pathogenesis of human breast carcinoma.

It has been demonstrated in preliminary studies that PRLr is present within the nucleus of malignant mammary epithelial cells as well as ductal and invasive ductal carcinomas. In addition, enhanced PRLr nuclear localization can potentiate Stat5a-mediated gene transcription. As such, the identification that nuclear PRLr can enhance the actions of Stat5a may serve to mediate the larger function of proliferation, survival and progression of

Figure 5. Nuclear PRLr is derived from the cell surface. Cells surface biotinylation and co-immunoprecipitation in T47D cells reveals that PRL stimulation triggers the intranuclear accumulation of PRLr. Calnexin is shown as a control for cytoplasmic contamination and the β1 integrin demonstrates a cell surface protein that is not nuclear localized.

breast cancer. This aim will test the central hypothesis that nuclear PRLr contributes to the progression of human breast cancer.
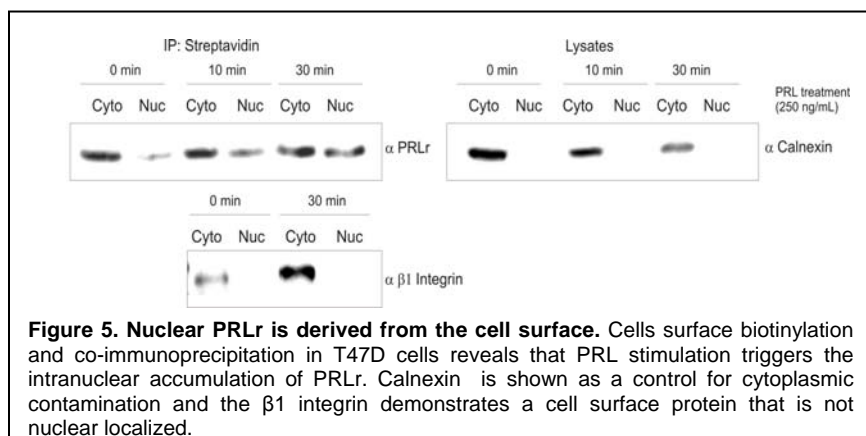
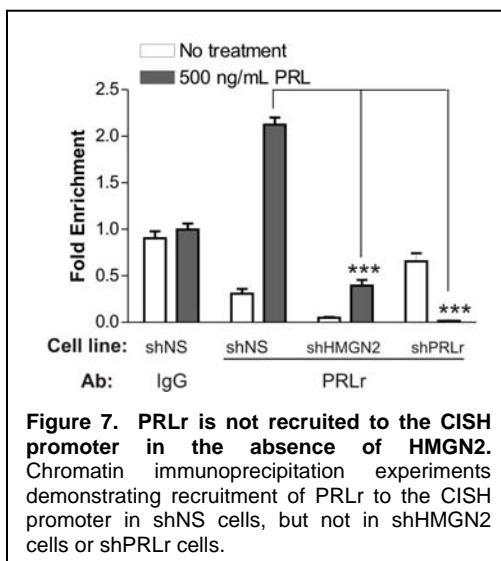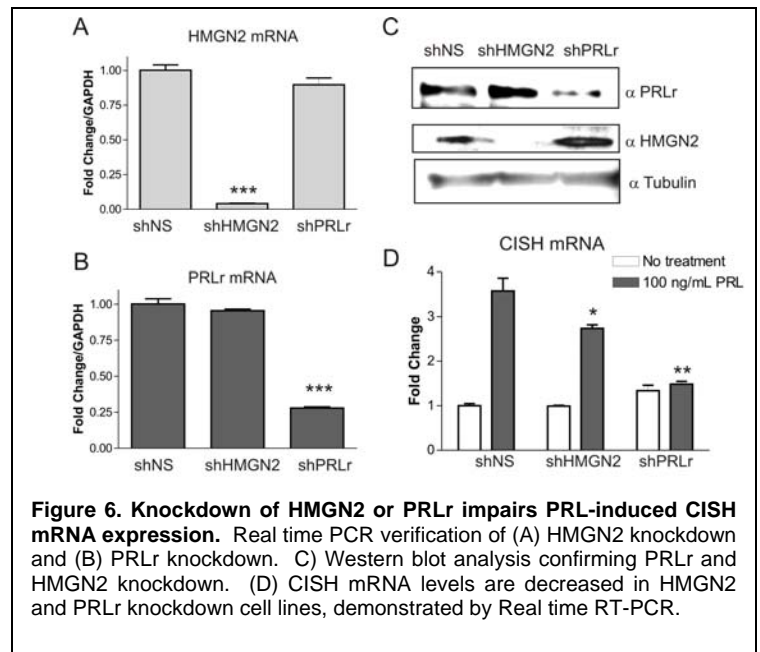## Creation of T47D stable knockdown cell pools, shHMGN2 and shPRLr

We have determined that HMGN2 is recruited to the CISH promoter by the PRLr. Based on this data, we postulated that HMGN2 knockdown would produce a similar phenotype to PRLr knockdown.

We obtained an shRNA targeting HMGN2 from origene, and designed an shRNA targeting the 3'UTR of PRLr. Knockdown results are shown in Figure 6 (A,B and C).

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When these cell lines were analyzed for their ability to activate CISH mRNA expression, knockdown of HMGN2 and PRLr both significantly decreased CISH mRNA levels.

To determine if PRLrwas being recruited to the CISH promoter ChIP was performed utilizing the generated stable knockdown cell pools shown in Figure 6. Unexpectedly, knockdown of HMGN2 prevented PRLr recruitment to the CISH promoter. We therefore postulate that HMGN2 might be required to stabilize PRLr binding on the CISH promoter. Similarly, PRLr may be required to HMGN2 recruitment and binding to the CISH promoter.

Therefore, future ChIP studies will determine if PRL is necessary for HMGN2 docking to the CISH promoter. We also plan on determining what marks of transcriptional activation are impaired in shHMGN2 cells.

Figure 6. Knockdown of HMGN2 or PRLr impairs PRL-induced CISH mRNA expression. Real time PCR verification of (A) HMGN2 knockdown and (B) PRLr knockdown. C) Western blot analysis confirming PRLr and HMGN2 knockdown. (D) CISH mRNA levels are decreased in HMGN2 and PRLr knockdown cell lines, demonstrated by Real time RT-PCR.

Figure 7. PRLr is not recruited to the CISH promoter in the absence of HMGN2. Chromatin immunoprecipitation experiments demonstrating recruitment of PRLr to the CISH promoter in shNS cells, but not in shHMGN2 cells or shPRLr cells.

Creation of stable overexpression cell pools

We have generated stable PRLr knockdown cell pool that targets the PRLr 3'UTR. Utilizing stable cell pools, we plan on performing rescue experiments by re-introduce either wt PRLr or transactivation deficient Y406FD411APRLr into shPRLr cells. Figure 8 shows epitope tagged PRLr expression for both wt and mutant PRLr. Future experiments will utilize these cell lines to examine both the mechanism of functional consequences of trasactivation deficient PRLr.

Figure 8. Epitope tagged PRLr expression in overexpression cell lines
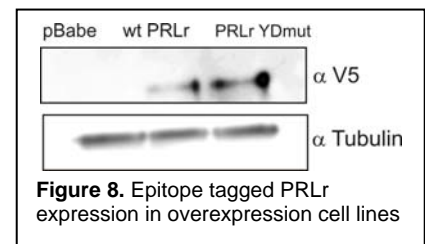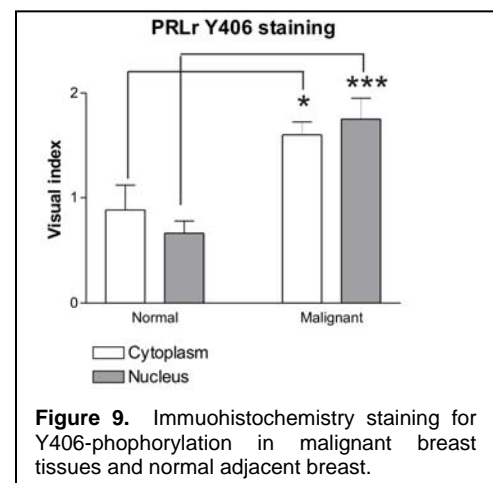
Translational implications of Nuclear PRLr

Data from our lab has demonstrated that one of the critical residues in PRLr transactivation, Y406, is phosphorylated. A phospho-specific antibody was therefore developed against Y406 to determine where this phosphorylation might occur, and how this phosphorylation might be related to breast cancer progression. A breast cancer and normal adjacent tissue was stained via immunohistochemistry using the phosphospecific PRLr Y406 antibody. In this study total PY406 staining increased in malignant tissue, and a majority of this staining was localized to the nucleus(Figure 9). This data suggests that phosphorylation of Y406 may be crucial for PRLr transactivation, and may initiate or contribute to a tumorigenic phenotype.

Part V - Key Research Accomplishments

- Identification of critical residues in the PRLr transactivation domain
- Verification that HMGN2 binds to the PRLr and is recruited to the CISH promoter upon PRL stimulation
- Cloning of several retroviral constructs, and development of expertise in retroviral transduction

Figure 9. Immuohistochemistry staining for Y406-phophorylation in malignant breast tissues and normal adjacent breast.

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- Providing key insight into the role of the PRLr as a transcriptional coactivator
- Providing evidence for the mechanism of PRLr nuclear localization
- Development of presentation skills through numerous departmental and cancer center wide talks
- Development of collaboration skills by learning techniques perfected by adjacent laboratories
- Development of mentor skills by assisting in colleagues paper revisions and by teaching new techniques

Part VI - Reportable Outcomes

Publications:

Fang F, Zheng J, Galbaugh T, **Fiorillo A**, Hjort E, Zeng X, Clevenger C. J Mol Endocrinol. 2010 Mar 17. Cyclophilin B as a co-regulator of prolactin-induced gene expression and function in breast cancer cells.

Conference Abstracts:

- ***AACR Annual Meeting (2009) Denver, CO. Alyson A. Fiorillo***, *Samantha L. Gadd and Charles V. Clevenger. "Nuclear PRLr functions as a transcriptional activator.*
- ***Northwestern University CGSA Fall Poster Session (2008) Alyson A. Fiorillo***, *Samantha L. Gadd, Feng Fang and Charles V. Clevenger. "Nuclear PRLr functions as a transcriptional activator.*
- ***Endocrine Society Annual Meeting (2008) San Francisco, CA (Invited Poster Competition) Alyson A. Fiorillo***, *Samantha L. Gadd, Feng Fang and Charles V. Clevenger. "The Role of Nuclear Prolactin Receptor as a Transcriptional Activator."*
- ***Northwestern University, Lewis Landsberg Research Day Annual Symposium (2008) Chicago, IL***
  Alyson A. Fiorillo, *Samantha L. Gadd, Feng Fang and Charles V. Clevenger. "The Role of Nuclear Prolactin Receptor as a Transcriptional Activator."*
- ***Gordon Research Conferences, Prolactin and Growth Hormone (2008) Ventura Beach, CA***
  Alyson A. Fiorillo, *Samantha L. Gadd, Feng Fang and Charles V. Clevenger. "The Role of Nuclear Prolactin Receptor as a Transcriptional Activator."*

Part VII - Conclusions

The central goal of this proposal was determine the role of the PRLr in the nucleus. Over the past year the experiments performed have provided significant evidence that this role is to function as a transcriptional co-activator. We have also identified that HMGN2, a nucleosome binding protein, interacts with the PRLr, and is recruited to the PRLr-Stat5a chromatin complex on the CISH promoter. Since there is little data describing how HMGN proteins alter transcription or what proteins confer their promoter specificity, the study of how PRLr might tether HMGN2 to the CISH promoter is highly relevant to understanding its cellular function and mechanism of action. We have also demonstrated that transactivation deficient PRLr, Y406FD411A cannot activate Gal4 transcription, bind to HMGN2 or promote the full activation of the Stat5a-driven CISH reporter. As knockdown of HMGN2 yields a similar effect to that of PRLr Y406FD411A, our data suggests that both transactivation-competent PRLr and HMGN2 may be required for the full transcriptional activity of Stat5a. In addition, immunohistochemistry data implicates that phosphorylation of the Y406, a critical residue in PRLr transactivation, is amplified in the nucleus of malignant breast tissue. This data therefore implies that a functional PRLr transactivation domain may be a hallmark of a tumorigenic phenotype. Our proposed work and work to date encompassing a transcriptional, mechanistic and functional analysis of nuclear PRLr, is integral in defining the role of this potential transcriptional regulator in the initiation and progression of breast cancer.

Part VIII – Appendices

1   **Cyclophilin B as a Co-regulator of Prolactin-induced Gene Expression**

2   **and Function in Breast Cancer Cells**

3

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5   **Elizabeth E Hjort[3], Xianke Zeng[1], Charles V Clevenger[1]***

6

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Abstract

The effects of prolactin (PRL) during the pathogenesis of breast cancer are mediated in part though Stat5 activity enhanced by its interaction with its transcriptional inducer, the prolyl isomerase cyclophilin B (CypB). We have demonstrated that knockdown of CypB decreases cell growth, proliferation, and migration, and CypB expression is associated with malignant progression of breast cancer. In this study, we examined the effect of CypB knockdown on PRL signaling in breast cancer cells. CypB knockdown with two independent siRNA was shown to impair PRL-induced reporter expression in breast cancer cell line. cDNA microarray analysis was performed on these cells to assess the effect of CypB reduction, and revealed a significant decrease PRL-induced endogenous gene expression in two breast cancer cell lines. Parallel functional assays revealed corresponding alterations of both anchorage-independent cell growth and cell motility of breast cancer cells. Our results demonstrate that CypB expression levels significantly modulate PRL-induced function in breast cancer cells ultimately resulting in enhanced levels of PRL-responsive gene expression, cell growth and migration. Given the increasingly appreciated role of PRL in the pathogenesis of breast cancer, the actions of CypB detailed here are of biological significance.

33   **Introduction**

34   An increasing body of literature supports a significant role for the hormone prolactin

35   (PRL) in the pathogenesis of human breast cancer. Epidemiologic analysis has revealed that PRL

36   concentration is associated with an increased risk for breast cancer (Eliassen, et al. 2007;

37   Hankinson, et al. 1999; Tworoger, et al. 2007) particularly in post-menopausal women. These

38   clinical observations are further substantiated by the PRL transgenic mouse model, which

39   develops both ER+ and ER- mammary tumors after 12-18 months (Rose-Hellekant, et al. 2003;

40   Wennbo, et al. 1997; Wennbo and Tornell 2000). Several lines of evidence have also indicated

41   that PRL acts as both an endocrine and autocrine/ paracrine progression factor for mammary

42   carcinoma *in vitro* and *in vivo* in rodents and humans (Clevenger, et al. 1995; Das and

43   Vonderhaar 1997).

44   The effects of PRL are mediated by the interaction to its receptor (PRLr) (Clevenger, et

45   al. 2003). Binding of PRL activates the pre-dimerized PRLr (Gadd and Clevenger 2006) and

46   results in the activation of PRLr-associated signaling cascades such as Jak2/Stat5, Fyn/Src,

47   Shc/Grb2, Sos/Raf/MAPK, and Tec/Nek3/Vav/Rac (Clevenger et al. 2003), resulting in the

48   transactivation of PRL-responsive genes, that include cyclin D1, CISH, and β-casein (Brockman

49   and Schuler 2005; Fang, et al. 2008; Guyette, et al. 1979; Pezet, et al. 1999; Utama, et al. 2006).

50   The summation of these signaling events results in the terminal maturation of normal mammary

51   tissues (Maus, et al. 1999; Miller, et al. 2007) and contributes to the PRL induced growth (18-

52   20), and motility (Maus et al. 1999; Miller et al. 2007) of malignant breast cells and tissues.

53   Previous research in our laboratory had demonstrated that some of the actions of PRL are

54   directly mediated by the localization and function of this ligand within the nucleus (Clevenger, et

55   al. 1991; Rycyzyn, et al. 2000). Yeast two-hybrid screening was used to identify binding partners

56   involved in this process (Rycyzyn and Clevenger 2002; Rycyzyn et al. 2000) and identified that

57   the cis-trans peptidyl prolyl isomerase, cyclophilin B (CypB), was a binding partner required for

58   the nuclear translocation of PRL (Rycyzyn et al. 2000). Within the nucleus, the PRL-CypB

59   complex was found to function as an inducer for the latent transcription factor Stat5, through its

60   induction of the release of the Stat5 inhibitor, PIAS3 (Rycyzyn and Clevenger 2002). CypB has

61   also been demonstrated to be associated with malignant progression and regulation of genes

62   implicated in the pathogenesis of breast cancer (Fang, et al. 2009a).

63   The cyclophilins are members of a larger class of PPIase proteins widely expressed

64   throughout the body, known as the immunophilins that are targets for the immunosuppressive

65   agents FK506, cyclosporine A (CsA), and rapamycin (Fischer, et al. 1998; Kofron, et al. 1991).

66   Although classically thought to assist in protein folding, immunophilins also serve as signaling

67   switches via prolyl isomerization (Hunter 1998), regulating the activity of cell surface receptors

68   TGFβ (Huse, et al. 1999), tyrosine kinases (Brazin, et al. 2002; Zheng, et al. 2008), and

69   transcription factors such as c-Myb (Leverson and Ness 1998) and IRF4 (Mamane, et al. 2000).

70   In addition to its actions within the nucleus as a transcriptional inducer, CypB as a secreted

71   protein is also thought to serve as a ligand for the CD147 receptor, thereby regulating the

72   motility of cells expressing this receptor (Melchior, et al. 2008; Pakula, et al. 2007; Yang, et al.

73   2006; Yurchenko, et al. 2001). Indeed, a recent study revealed that CypB present in the

74   conditioned medium of the breast carcinoma cell line MDA-MB231 promoted chemotaxis of

75   bone marrow-derived mesenchymal stromal cells (MSCs) (Lin, et al. 2008). However, despite

76   these insights into CypB action, the function of CypB during PRL-induced gene expression and

77   action in breast cancer cells has remained undocumented.

4

78   In this study for the first time, the effects of altering CypB levels in breast cancer cells

79   during PRL stimulation were examined at the level of gene expression profiling, anchorage

80   independent growth, and cell migration. These studies demonstrate that expression levels of

81   CypB significantly augmented PRL-induced action in breast cancer cells ultimately resulting in

82   enhanced levels of growth and migration of breast cancer cells.

83

84   **Materials and methods**

85   **Cell lines, vectors and reagents**

86   The human breast cancer cell lines (T47D and MCF7) from American Type Culture

87   Collection (ATCC, Manassas, VA) were maintained in DMEM (Hyclone) supplemented with

88   10% fetal bovine serum (FBS), 50 µg/ml penicillin, and 50 µg/ml streptomycin in a humidified

89   atmosphere of 5% CO_2 at 37°C. The si-CypB cells (T47D siRNA CypB knockdown stable cells)

90   and si-Luc cells (siRNA control cells for non-genomic target) were described before (Fang et al.

91   2009a). Vectors used in this study are: firefly luciferase reporter pGL4-CISH (Fang et al. 2008),

92   pGL4-LHRE (Fang, et al. 2009b), renilla luciferase reporter pGL4.73 (Promega), pGL410-SV40

93   (Fang et al. 2009a), pcDNA3.1 (Invitrogen, Casbad, CA), and pcDNA3.1-CypB (Rycyzyn et al.

94   2000). Human recombinant prolactin was a gift from Dr Tony Kossiakoff (University of

95   Chicago). Antibodies used in this study are: anti-CypB (Invitrogen, 37-0600) and α-tubulin

96   (Zymed, 32-2500).

97   **Knockdown of CypB**

98   For these studies two separate knockdown approaches were utilized for cross-validation

99   purposes to exclude off target effects. The first approach used the lentiviral si-CypB sequence

100   (this siRNA sequence is against the 3'-end region of the CypB coding sequence) (Robida, et al.

101   2007). The si-CypB cells (T47D siRNA CypB knockdown stable cells) and si-Luc cells (siRNA

102   control cells for non-genomic target) (Fang et al. 2009a) were previously used before. For

103   independent validation of the effects of siRNA knockdown of CypB, a second approach used a

104   different siRNA (si-CypB-T, against the central region of the CypB coding sequence, Cat#: D-

105   001136-01-05, Dharmacon, Lafayette, CO) that was transiently transfected in T47D and MCF7

106   cells in parallel with si-control RNA (Cat# D-001210-01 from Dharmacon) using RNAiMAX

107   (Invitrogen). After 48 hours, cells were harvested for RNA isolation.

108   **Luciferase assay, Western blot, microarray and data analysis, RT-PCR and real-time PCR,**

109   **soft agar growth and cell motility assay (also in the supplemental methods)**

110   Dual luciferase assay was conducted according to Fang (Fang et al. 2008), and details are

111   described in the figure legends. Microarray was conducted on Illumina Human Ref-6 Version 2

112   Expression Chip (Illumina, San Diego, CA). T47D si-CypB cells were cultured in the growth

113   medium for three days followed by 24 hours arrest prior to PRL treatment (100 ng/ml) for 2

114   hours. RNA isolation for microarray analysis was conducted as described in Fang et al (Fang et

115   al. 2009a) and supplemental methods. Primers for real-time PCR are listed in the table in the

116   supplemental methods. Microarray data were deposited in Gene Expression Omnibus database

117   with accession number GSE15505 (GEO, http://www.ncbi.nlm.nih.gov/geo/). Soft agar growth

118   and cell motility assay were conducted according to Zheng et al (Zheng et al. 2008). The details

119   of methods are in the supplemental methods.

120   **Statistical analysis**

121   All experiments described here were performed at least three times. Statistical analysis

122   was performed on GraphPad Prism 4 (GraphPad Software, La Jolla, CA), and specified in the

6

123   figure legends. The results are shown as the means with error bars depicting +/- Standard Error

124   Mean (SEM). * denotes $p < 0.05$, ** denotes $p < 0.01$ and *** denotes $p < 0.001$. $p < 0.05$ is

125   considered as statistically significant.

126

127   **Results**

128   **The genes regulated by PRL contribute to essential cell functions**

129   In addition to its well-recognized regulation of genes associated with milk production and

130   lactation (Clevenger, et al. 2008), PRL has been shown to induce expression of many genes

131   involved in cell proliferation and survival including BCL2 (Beck, et al. 2002), CEBPβ (Nanbu-

132   Wakao, et al. 2000), CISH (Pezet et al. 1999; Utama et al. 2006), c-Myc (Dominguez-Caceres, et

133   al. 2004), and cyclin D1 (Brockman, et al. 2002). To systematically query the effects of PRL on

134   global gene expression in human breast cancer cells, the estrogen receptor positive (ER+) T47D

135   breast cancer cell line was used for microarray analysis with or without PRL treatment. Two

136   hours of PRL stimulation (100 ng/ml) was selected as the optimal time course length, given the

137   preliminary data from our lab that had shown maximal RNA expression from the PRL-

138   responsive CISH, c-Myc and cyclin D1 gene loci at that time (Fang et al. 2009b). RNA isolated

139   from these cells was subject to cDNA synthesis, labeling, and hybridization to the Illumina

140   human cDNA chip. In order to reduce false positives, probes with measurement value below the

141   background level (detection p-value < 0.01) in all hybridizations were filtered out. 17901 probes

142   were kept for subsequent statistical analysis. To establish a physiologically relevant cut-point for

143   analysis of PRL-induced gene expression, the RNA levels of the PRL-induced c-Myc gene

144   (Dominguez-Caceres et al. 2004) was assessed. While microarray analysis demonstrated a 1.4-

145   fold induction of c-Myc expression, real-time PCR revealed that c-Myc was induced 2.4-fold

146   following PRL treatment. Similar phenomenon was also observed for BCL3, CEBPβ, CISH, and

147   cyclin D1 (see Fig 5). In addition, as documented by the manufacturer, the Illumina cDNA array

148   is sensitive to detect as little as 1.3-fold change in either direction (up or down). Given this, the

149   criterion to filter the microarray data was set to 1.3-fold (up or down regulation) for subsequent

150   analysis.

151   To simultaneously indicate the size biological effects (log2 fold change as X-axis) and

152   the statistical significance (-log10 p-value as Y-axis) at global level, a volcano plot was used to

153   compare the difference of gene expression between two groups (Fig 1A). As seen in volcano

154   map, the red dots represent the selected differentially expressed genes significantly regulated by

155   PRL treatment ($p<0.05$, FDR<0.05, fold change≥1.3 up or down). Two-dimensional hierarchical

156   clustering was applied to these filtered probes to generate a global overview of gene expression

157   map in the form of a heat map. Heat map analysis showed a remarkable difference in gene

158   expression pattern between PRL treatment and non-PRL treatment groups. It also indicated, in a

159   global view, highly consistent results among the triplicates in each group (Fig 1B). Of 28

160   significantly differentially expressed genes, 19 genes were up-regulated and 9 genes were down-

161   regulated. Many genes listed therein have not been reported upon before as PRL-responsive

162   genes in breast cancer, including BCL3 and BCL6 (Fig 1B). Subsequent real-time PCR analysis

163   confirmed the PRL-regulated mRNA upregulation of BCL3 expression and downregulation

164   BCL6 expression (Fig 1C and 1D). BCL3 plays an important role in cell proliferation (Na, et al.

165   1999), and sequence analysis using TFsearch program revealed 10 Stat binding sites within the

166   10 kb region including promoter/enhancer upstream of transcriptional start point, as well as

167   within the exons, introns and the 3' untranslated region. BCL6 is a zinc finger transcription

168   factor and found to be expressed in the breast cancer cells (Logarajah, et al. 2003), and

169   overexpression of Stat5 has been found to repress BCL6 expression (Scheeren, et al. 2005;

170   Walker, et al. 2007). Interestingly, the regulation of menin expression via PRL regulation of

171   BCL6 regulates pancreatic ß-cell growth in pregnant mice; loss of this process may contribute to

172   gestational diabetes mellitus (Karnik, et al. 2007).

173   Ingenuity Pathway Analysis (IPA) has been widely used for analysis of gene expression,

174   proteomics and metabolic data to elucidate tumor progression, biomarker discovery and drug

175   discovery (Ganter and Giroux 2008). To assess the global effects of PRL on gene expression,

176   IPA was used to systematically visualize the relationships of genes regulated by PRL. The

177   transcripts from microarray results were filtered and 28 transcripts were input into the IPA Base

178   (see Material and methods). IPA was used to overlay the PRL-regulated genes onto the global

179   networks developed from the information contained in the Ingenuity Pathways Knowledge Base.

180   The genes regulated by PRL were categorized into different biological functions, with the

181   "cancer" biological function shown as the most significant category (Fig 1E). When the 28

182   transcripts were the mapped into the IPA Base, the most significant modulated interaction

183   network was associated with those genes implicated in the pathogenesis of "cancer,

184   hematological disease and cell cycle" (Fig 1F). This interaction network revealed that many of

185   these PRL-regulated genes interact with each other, and in doing so may enhance PRL-induced

186   tumorigenesis. Network analysis of these cancer associated genes reveals a central role for many

187   gene products involved in PRL-signaling, such as CISH, MAPK, CEBPβ, ERK and c-Myc.

188   **Ectopic expression of CypB enhanced Stat5-responsive reporter expression**

189   PRL action is mediated in the cells through distinct signaling pathways including the

190   Jak2/Stat5 signaling pathway. In this pathway, the intranuclear PRL/CypB complex acts as a

191   Stat5 transcriptional inducer to regulate gene expression (Rycyzyn and Clevenger 2002;

192   Rycyzyn et al. 2000). To characterize the role of CypB during PRL-induced expression of Stat5-

193   responsive genes in breast cancer cells, reporter assay was used to test the effect of CypB ectopic

194   expression on such promoters in T47D cells. This cell line was selected for our initial screening

195   studies given its regulatory robust expression of both the PRLr and CypB (data not shown). For

196   these studies, the pGL4-CISH luciferase reporter was used for PRL-induced reporter assay (Fang

197   et al. 2008); this expression construct contained the 1kb CISH promoter region fused upstream of

198   firefly luciferase reporter gene. These results showed that ectopic expression of CypB itself had

199   no effect on luciferase expression of the pGL4-CISH reporter in the absence of PRL (Fig 2A). In

200   the presence of PRL, CypB enhanced PRL-induced luciferase expression of pGL4-CISH reporter

201   (Fig 2A). As a parallel control experiment, we also co-transfected pGL4-CISH with the

202   pcDNA3.1-CypB-PPIase construct (a CypB mutant construct lacking activity of peptidyl-prolyl

203   cis/trans isomerase (PPIase or PPI)) in T47D cells. Results showed that compared to non-PRL

204   treatment, PRL-induced luciferase fold change is 22.5 ± 3.3 and 22.0 ± 3.0 respectively in

205   pcDNA3.1 and pcDNA3.1-CypB-PPIase transfectants, suggesting that the CypB-mediated

206   enhancement of PRL-induced luciferase expression was dependent upon PPI activity. These

207   results confirmed the effects of CypB on PRL-induced gene expression (Rycyzyn and Clevenger

208   2002) in T47D breast cancer cells.

209   **Knockdown of CypB impaired the expression of Stat5-responsive reporters**

210   To complement the overexpression-based studies, the effects of CypB knockdown on the

211   PRL/Stat5 signaling pathway were assessed in T47D transfected stably with si-RNA against a

212   control (luciferase; termed si-Luc) or CypB (termed si-CypB) (Fang et al. 2009a). Results from

213   real-time PCR and microarray confirmed a significant knockdown of CypB (Fig 2B). A second

214   different sequence directed against CypB (termed as si-CypB-T, and control as si-control-T) was

10

215   transiently transfected into T47D and MCF7 cells to validate that the effects noted in siCypB

216   stable transfectant were not due to off-target action (Fang et al. 2009a). The effect of transient

217   transfection of this siRNA also resulted in significantly reduced CypB levels (Fig 2C). This

218   second si-RNA was also used later in gene profile validation studies (see Fig 3). Two PRL-

219   induced, Stat5 responsive promoter reporter constructs, termed pGL4-LHRE (a synthetic

220   construct containing six Stat5 responsive elements) and pGL4-CISH were then introduced into

221   the stable T47D si-CypB cells to evaluate the effects of CypB knockdown on PRL signaling.

222   Results (Fig 2D) showed that, compared to wild type cells, the luciferase expression of pGL4-

223   CISH in si-CypB cells had little change in the absence of PRL. In the presence of PRL, the

224   luciferase expression of pGL4-CISH in si-CypB cells decreased significantly compared to wild

225   type cells. Similar results were observed for pGL4-LHRE (Fig 2E). These findings revealed that

226   CypB knockdown decreased PRL-induced expression luciferase in si-CypB cells, further

227   demonstrating the potentiating effects of CypB on PRL-mediated gene expression.

228   **Knockdown of CypB impaired the PRLr and PRL-inducible endogenous gene expression**

229   To determine the effects of CypB knockdown on PRL-regulated gene expression,

230   microarray analysis was conducted using si-CypB cells treated with or without PRL. To

231   characterize the effect of CypB on PRL signaling in this analysis, "prolactin" was used as the

232   key word in the "Gene Ontology (GO)" program to search for PRL-related genes significantly

233   regulated by CypB knockdown. Results revealed that the expression of the prolactin receptor

234   (PRLr), S100A6, and PIP (the prolactin-inducible protein) were significantly altered in si-CypB

235   cells compared to si-Luc cells (Fig 3A), a finding further confirmed by both real-time PCR and

236   Western blot analysis for PRLr gene (Fig 3B and 3C).

11

237   Microarray analysis and subsequent validation studies suggested that the panel of the

238   PRL-induced genes was regulated differently by PRL in si-Luc and si-CypB cells. Real-time

239   PCR confirmed that CEBPβ, c-Myc, and cyclin D1 mRNA expression were significantly

240   decreased in si-CypB stable cells compared to that of si-Luc stable cells, while little effect on

241   BCL3 and CISH was observed (Fig 4A-E). Real-time PCR results also showed that the PRL-

242   repressed BCL6 mRNA expression was also significantly decreased in si-CypB cells compared

243   to that of si-Luc cells (Fig 4F). The effect of transient transfection of a second differing siRNA

244   (used in Fig. 2E, termed as si-CypB-T, and control as si-control-T)) into both T47D and MCF7

245   cells was also tested in the context of PRL to further rule out off target effects. The PRL-induced

246   CEBPβ and cyclin D1 mRNA expression were significantly decreased in cells with CypB

247   knockdown (Fig 4G-L). While si-CypB-T cells resulted in a decrease in c-Myc and BCL6 levels,

248   these results did not achieve statistical significance compared to results from the siCypB stable

249   T47D cells. This may be due to cell line varience (high PRLr level in T47D and modest PRLr in

250   MCF7) and difference in knockdown efficiency (stable vs transient). Taken together, these

251   findings indicate that reductions in CypB levels significantly impacted on PRL-induced gene

252   expression at both global and locus-specific levels.

253   **CypB knockdown impaired the PRL-induced colony growth on the soft agar**

254   Given that CypB knockdown significantly modulated the expression of the PRL-

255   regulated CEBPβ, c-Myc, cyclin D1 and BCL6 genes in T47D cells, the effect of CypB

256   knockdown on PRL-induced anchorage-dependent cell growth was tested using soft agar assay.

257   These analysis revealed colony number (counted by single colonies) and size (determined by the

258   colony area) of si-Luc cells were increased in the presence of PRL. Colony number and size of

259   si-CypB cells was significantly attenuated, showing that CypB is involved in the anchorage-

260   independent cell growth (Fig 5A-F).

261   **CypB knockdown impaired the PRL-induced cell motility**

262   PRL has also been shown to stimulate T47D cell motility (Maus et al. 1999; Miller et al.

263   2007). To investigate if CypB knockdown affected PRL-induced cell motility, Boyden chamber

264   cell migration assay was conducted using PRL as a chemoattractant. These data revealed that

265   PRL stimulated si-Luc cell motility, as previously described (Miller et al. 2007). The PRL-

266   induced motility of the si-CypB cells were significantly decreased, revealing a contribution of

267   CypB to PRL-mediated cell motility (Fig 6). In our previous publication (Fang et al. 2009a), we

268   have shown that knockdown of CypB impaired the FBS-inducible and estradiol-inducible cell

269   migration, suggesting the impairment of cell motility in si-CypB cells is due to global effects on

270   gene expression involved in motility regulation. It is also noted that CypB knockdown decreased

271   migration and colony formation even without PRL. This input may be due to the inhibition of the

272   actions of autocrine PRL produced by these cells. In the presence of PRL, CypB knockdown

273   impaired PRL-induced migration and colony formation (Fig 5 and 6).

274

275   **Discussion**

276   Given the increasingly appreciated role of PRL in the pathogenesis of breast cancer,

277   surprisingly little is known in regards to it regulation of gene expression in such cells. In this

278   context, this study sought to examine the effects of both PRL stimulation and CypB knockdown

279   on PRL-induced gene expression by validated gene profiling and relate these observations to

280   PRL-induced growth and motility. CypB has been shown previously to play an important role in

13

281   cell survival (Kim, et al. 2008), cancer progression, cell proliferation and cell growth (Fang et al.

282   2009a). Our results indicate that absence of CypB significantly impair the patterns of gene

283   expression induced by PRL in breast cancer cells.

284   Gene profiling analysis performed here and elsewhere (Fang et al. 2009a) showed that

285   CypB knockdown regulated PRL-related genes included: 1), the PRLr. It is important to note that

286   given widely varying effects of CypB knockdown on PRL-induced genes (see Fig 3), we believe

287   that the effects of CypB extend beyond that of a mere reduction in PRLr levels, 2) the prolactin-

288   inducible protein mRNA (PIP or GCPD15); this protein was noted to be increased two-fold in si-

289   CypB cells. PIP was first cloned from T47D cells and was induced by five days treatment of

290   PRL and/or growth hormone (Murphy, et al. 1987). Higher mRNA levels of PIP has a high

291   correlation with the expression of estrogen receptor alpha (ERα), progesterone receptor (PR) and

292   low-grade tumours (Clark, et al. 1999), 3) mRNA for the prolactin regulatory element binding

293   protein (PREB, also known as SEC12 or MGC3467); this mRNA was up-regulated by PRL only

294   in si-CypB cells (not in si-Luc cells). PREB acts as a transcriptional activator on PRL promoter

295   region to regulate PRL expression (Fliss, et al. 1999), 4) S100A6 mRNA; this is a S100 calcium

296   binding protein, also known as prolactin receptor-associated protein, and is down-regulated in si-

297   CypB cells (Fang et al. 2009a). S100A6 has been observed in prolactin receptor

298   immunoprecipitates (Murphy, et al. 1988). S100A6 is up-regulated in breast cancer cells and

299   tissues (Maelandsmo, et al. 1997) and knockdown of this gene appears to decrease both cell

300   proliferation and motility (Breen and Tang 2003), 5) the c-Myb proto-oncogene mRNA; this

301   oncogene was downregulated in PRL-stimulated si-CypB cells (p<0.05). c-Myb is associated

302   with cell differentiation and proliferation (Weston 1999). c-Myb protein level is increased in *in*

303   *situ* and invasive breast cancers (McHale, et al. 2008). The c-Myb mRNA levels were regulated

14

304   by cyclophilin isomerase activity (Leverson and Ness 1998). c-Myb is a Stat5a co-activator

305   during PRL/Stat5a-driven gene expression (Fang et al. 2009b), and 6) the CEBPβ transcription

306   factor; the level of its mRNA was also downregulated in si-CypB cells. CEBPβ is bZIP

307   transcription factor acting as a Stat5 co-activator and is also a Stat5-regulated gene (Nanbu-

308   Wakao et al. 2000). The PRLr promoter has CEBPβ binding sites and overexpression of CEBPβ

309   upregulates PRLr expression (Hu, et al. 1998). This suggests CypB has a profound impact on

310   PRL-related gene expression.

311   Our previous work using a matched, progressive-based breast tissue microarray (TMA)

312   (Fang et al. 2009a) showed that CypB levels were increased in malignant breast epithelium,

313   suggesting that the up-regulation of CypB in breast cancer could significantly modulate the

314   biology of this disease. Si-RNA-mediated knockdown of CypB was found by gene profiling to

315   significantly regulate genes related cell proliferation, motility and receptors (Fang et al. 2009a).

316   Since CypB mediates PRL-responsive gene expression with profound effects, we hypothesized

317   that CypB is multi-faceted, serving as: 1) an activator of receptor expression (i.e. PRLr), 2) a

318   chaperone for ligand (i.e. PRL), and 3) an inducer for the transcriptional factor (i.e. Stat5)

319   (Rycyzyn and Clevenger 2002), all of which may collectively contribute to the regulation of the

320   PRL-responsive genes. Given the significant effect of CypB knockdown on the PRL-induced

321   anchorage-independent cell growth and cell motility, these results would suggest that the

322   molecular actions of CypB within the cell during PRL-induced signaling impacts significantly on

323   the biology of breast cancer cells.

324   The precise role of Stat5 in the pathogenesis of breast cancer remains to be fully clarified.

325   While data from mouse models of mammary cancer clearly indicate that loss of Stat5 function

326   results a significant delay in the pathogenesis of malignancy at this site (Ren, et al. 2002), data

327   from human tissues has shown that phosphorylated/nuclear Stat5 is associated with a favorable

328   histopathology (Sultan, et al. 2005). Although CypB could influence Stat5 function both through

329   its indirect actions at the cell surface, or by its direct interaction with Stat5, it is interesting to

330   note that many of the gene transcripts influenced by reduction in CypB expression in this

331   manuscript demonstrate multiple Stat5-binding sites within their proximal promoter regions. As

332   such, these findings would suggest that inhibitors that target both PRL-induced signals and CypB

333   may have a synergistic potential at the level of Stat5 function in the treatment of breast cancer.

334

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492

493   **Figure legends**

494   Fig. 1. Microarray analysis demonstrates the effects of PRL on global gene expression. A.

495   Volcano map demonstrates the relationship between the observed fold change in gene expression

496   and the P-value significance of such changes in PRL-treated cells. The dotted lines represent the

497   p-value and fold-change cut-offs. The red dots represent the selected genes filtered by criteria

498   (fold change $\geq$1.3 up or down, p<0.05, FDR<0.05). B. Heat map analysis reveals a global view

499   of genes up- and down-regulated in PRL-treated cells. C and D. Real-time PCR validates

500   microarray results for BCL3 (C) and BCL6 (D). Statistical analysis was performed using Student

501   t test. E. The biological functional categories were obtained from the Molecular and Cellular

502   Function in the IPA database. F. The top interaction networks generated using IPA analysis

503   included genes associated with "cancer, hematological disease and cell cycle". The color

504   indicates genes up-regulation (red), down-regulation (green) and complexes (gray).

505

506   Fig. 2. The effects of CypB overexpression and CypB knockdown on a Stat5-responsive reporter.

507   A. Luciferase assay using pGL4-CISH reporter. Cells were transfected with pGL4-CISH

508   reporter, the renilla luciferase control (pGL4.73) and pcDNA3.1-CypB expression vector.

509   Transfectants were cultured in the minimal defined medium for 24 hours followed by 24 hours of

510   PRL stimulation prior to luminescence assay. B and C. CypB knockdown in T47D cells

511   confirmed by real-time PCR and microarray (B), and transient transfection (C). D and E.

512   Luciferase assay using pGL4-CISH (D) and pGL4-LHRE (E). T47D parental cells (wt) or si-

513   CypB cells were co-transfected with 100 ng of pGL4-CISH (D) or pGL4-LHRE (E), along with

514   2 ng of renilla luciferase control (pGL4.73) and 400 ng of pcDNA3.1-CypB expression vector,

515   and maintained in the FBS-containing growth medium overnight. Transfectants were then

21

516   starved in the FBS-free minimal defined medium for 24 hours followed by 24 hours of PRL

517   (10ng/ml for pGL4-CISH and 100ng/ml for pGL4-LHRE) stimulation prior to luminescence

518   assay. Statistical analysis was performed using two-way ANOVA.

519

520   Fig. 3. The characterization of PRL-related genes affected by CypB knockdown. A. Heatmap

521   showed downregulation of PRLr, S100A6 and PIP in si-CypB cells compared to si-Luc control

522   cells. B and C. Real-time PCR (B) and Western blot (C) confirmed the PRLr downregulation in

523   si-CypB cells. Statistical analysis was performed using Student t test.

524

525   Fig. 4. Real-time PCR validated the impairment of PRL-induced gene expression by CypB

526   knockdown. A-F in T47D si-CypB stable cells. G-H in MCF7 cells with transient transfect of si-

527   CypB-T. The y-axis label "Fold change" is defined in the materials and methods section.

528   Statistical analysis was performed using Student t test.

529

530   Fig. 5. CypB knockdown results in the decreased PRL-induced soft agar growth and cell

531   motility. Cells were grown on soft agar for two weeks, and the pictures were taken under phase

532   contrast microscopy. A, si-Luc without PRL treatment. B, si-Luc with PRL treatment (200

533   ng/ml). C, si-CypB without PRL treatment. D, si-CypB with PRL treatment (200 ng/ml). E,

534   colony number on the soft agar. F, the total colony area on the soft agar.

535

536   Fig. 6. CypB knockdown results in the decreased PRL-induced cell motility. Cell motility was

537   assayed using Boyden chamber migration assay. The inserts were coated with collagen I

22

538   overnight. T47D cells were arrested in the FBS-free medium and placed in the inserts. Cells were

539   cultured for 20 hours and the migrated cells were counted under microscope. Statistical analysis
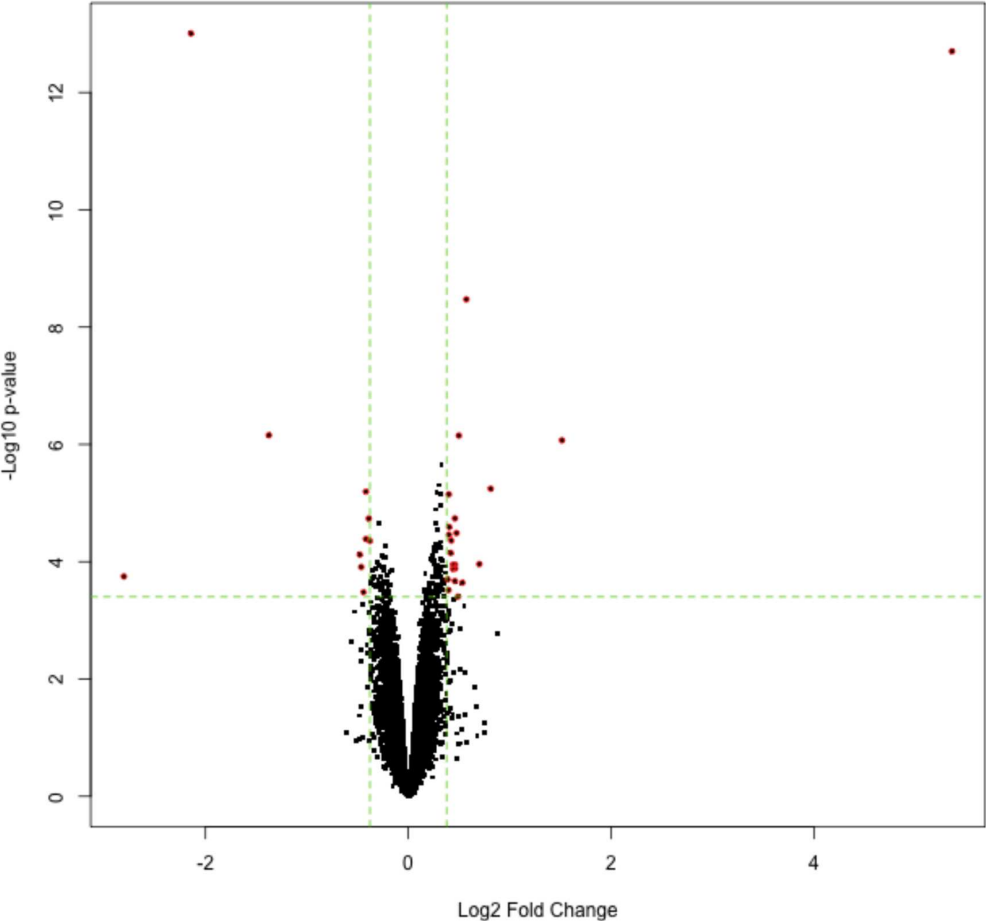
540   was performed using two-way ANOVA.
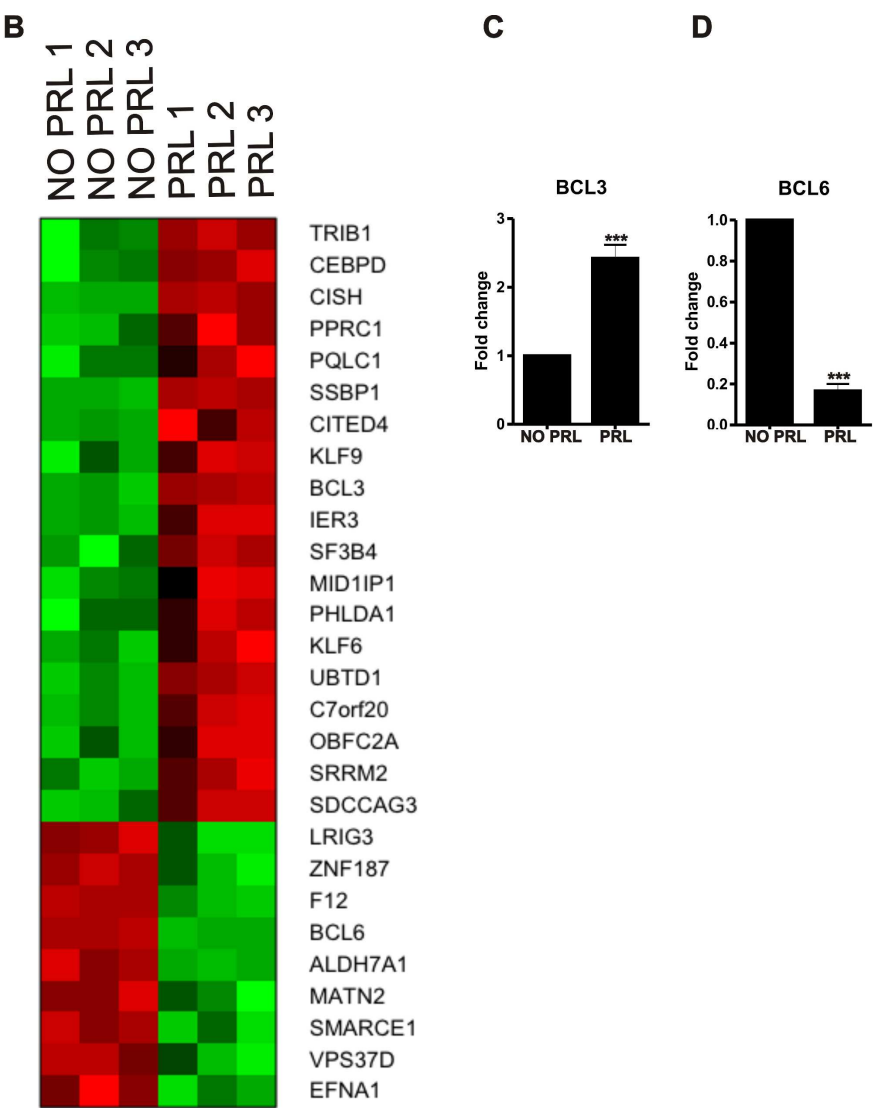
A

Fig1

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Fig1
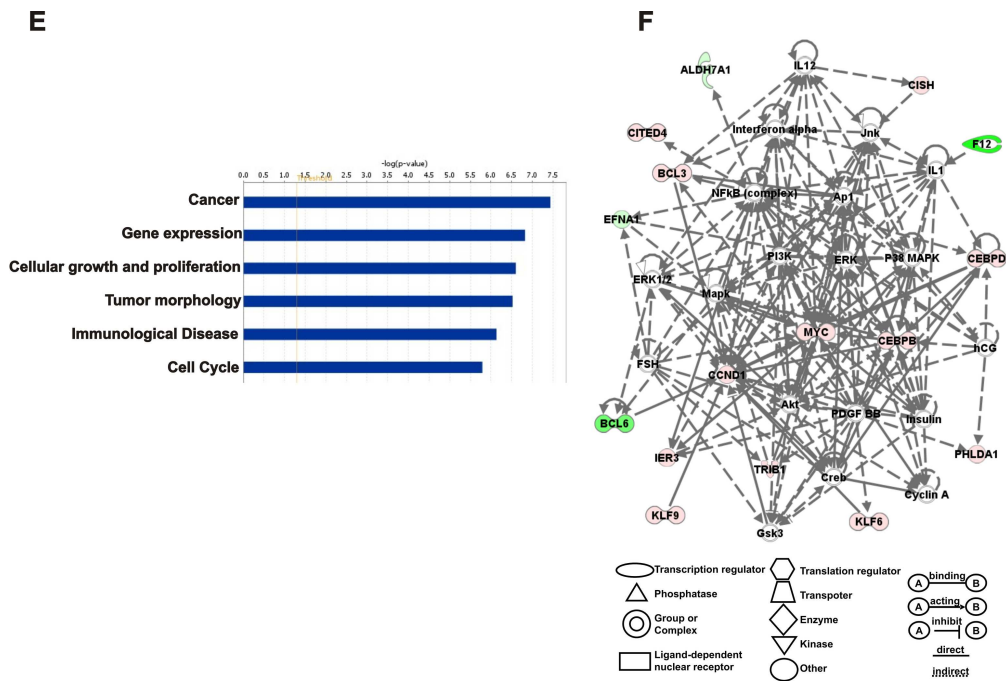
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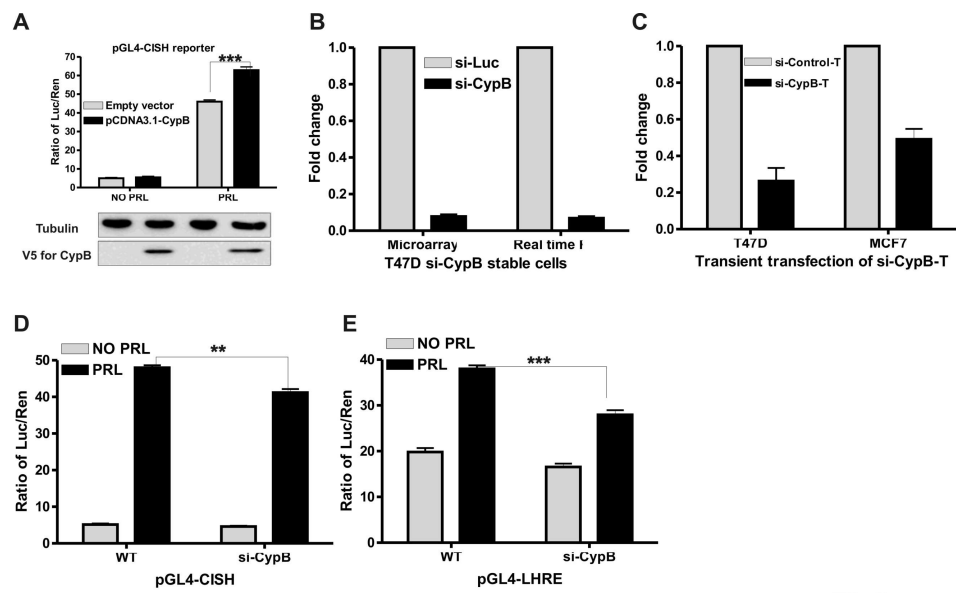
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F

Fig1

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Fig2

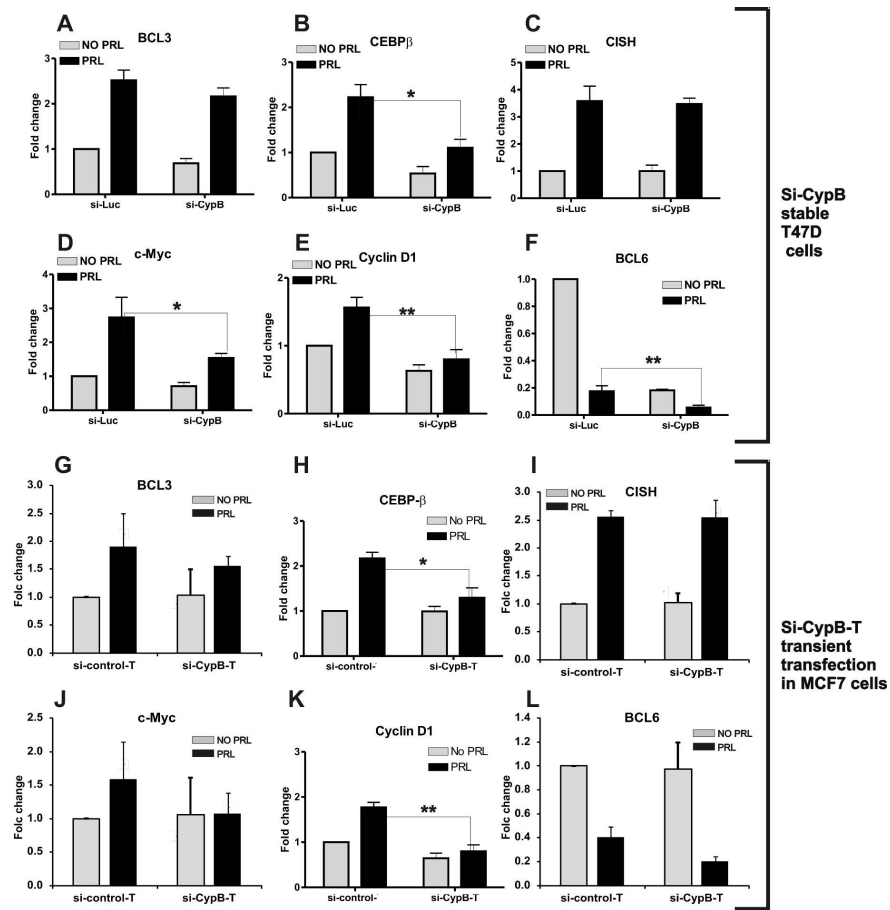117x71mm (600 x 600 DPI)
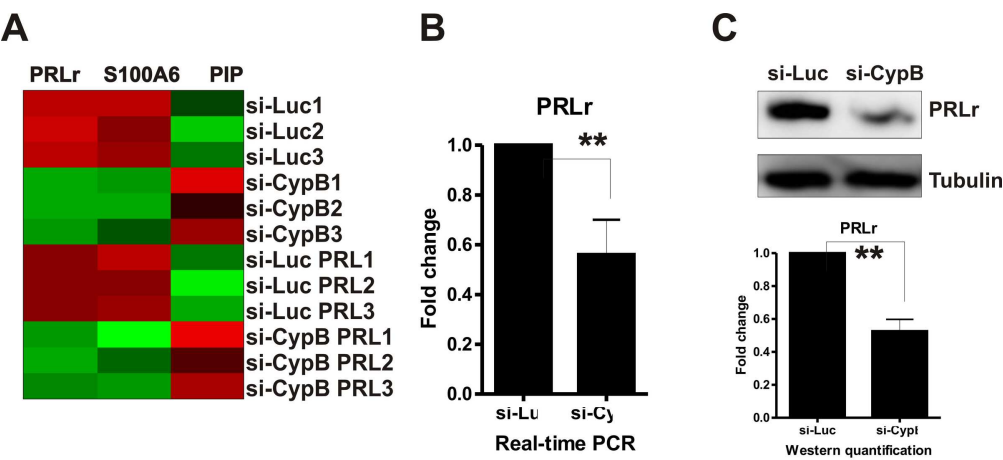
Fig3

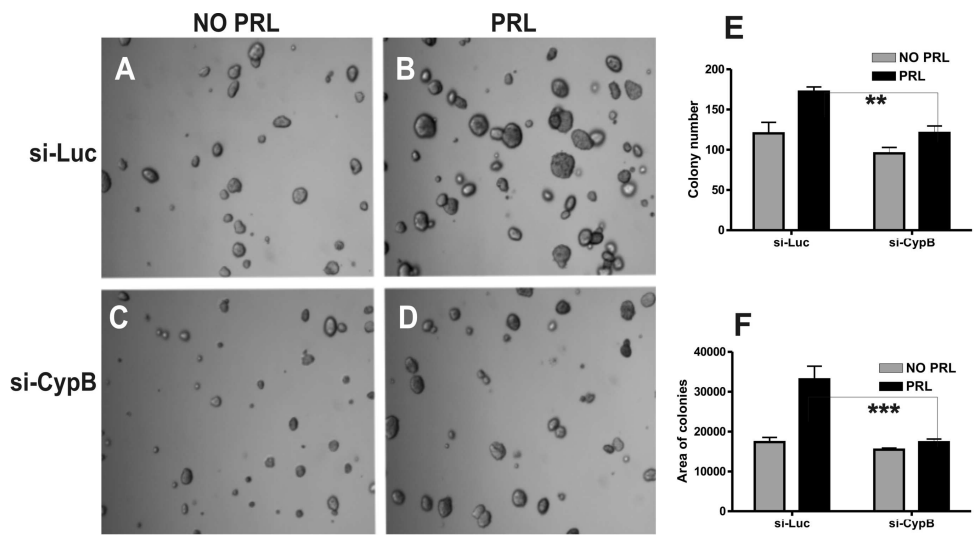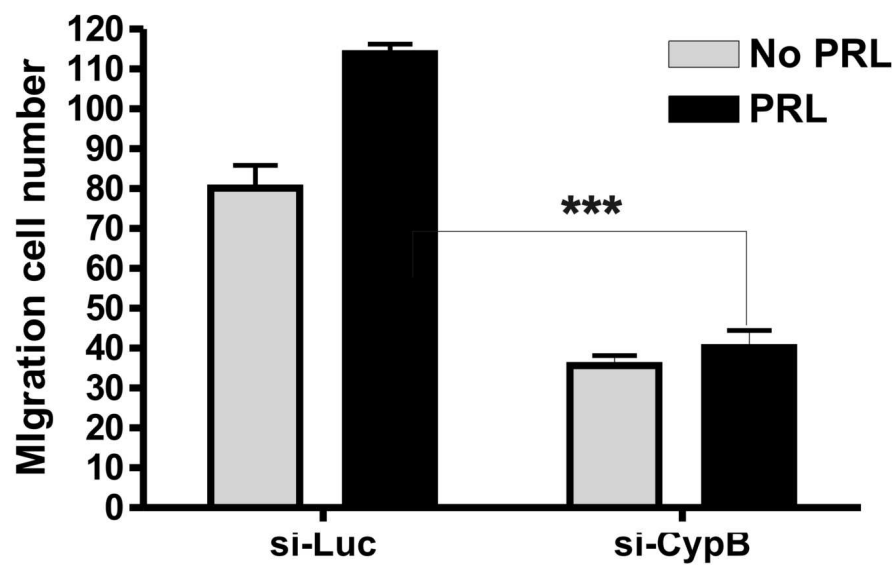101x59mm (600 x 600 DPI)

Fig4

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Fig5

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Fig6

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